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Histochemistry of gingiva: A review article

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Abstract
Histochemistry is the study of identification and distribution of chemical compounds within and between biological cells using histological techniques such as histological stains, indicators and light [optical] and electron microscopy. Histochemistry combines the methods of histology with those of chemistry or biochemistry, to reveal the biochemical composition of tissues and cells. This review discusses the histochemistry of gingiva to provide useful information regarding the chemical and enzyme systems of normal gingiva. In addition to adding to our understanding of physiologic processes in the gingiva this information provides the guidelines for interpreting the changes which occur in gingival diseases.

Keywords: histochemistry, gingiva, histological stains, electron microscopy

1. Introduction
Histochemistry is the study of identification and distribution of chemical compounds within and between biological cells using histological techniques such as histological stains, indicators and light [optical] and electron microscopy. It involves: Histochemistry, Enzyme histochemistry, Immunocytochemistry, In situ hybridization.

1.1 Basic Principles of Histochemistry
Histochemistry combines the methods of histology with those of chemistry or biochemistry, to reveal the biochemical composition of tissues and cells beyond the acid-base distribution shown by standard staining methods (Hx & E), without disrupting the normal distribution of the chemicals.
This review discusses the histochemistry of gingiva to provide useful information regarding the chemical and enzyme systems of normal gingiva.

2. Applications
- Identify, quantify, and localize chemical substances, gene expression, biological structures, organelles, specific cell types
- Clarify cell and tissue structure and morphology.
- Demarcate functional boundaries.

2.1 Limitations of the Current Methods
- Cannot be used for real time in vivo analysis of any tissue (requires the removal and killing of the tissue).
- Uses in humans limited to biopsied tissues.
- For looking at changes in tissue over time, each point in time requires a new tissue sample from a new animal.
- Tissue preparation and histo-chemical analysis may alter specimen morphology or chemistry depending on the methods and materials used.

2.2 Goal of Histochemistry
- **Presentation of normal chemical distribution:** The substance being analyzed must not diffuse away from its original site.
- **Presentation of normal chemical composition:** The procedure must not block or denature the reactive chemical groups being analyzed, or change normally non-reactive groups into reactive groups.

- **Specificity of the Reaction:** The method should be highly specific for the substance or chemical groups being analyzed, to avoid false-positive results.
- **Detectability of the Reaction Product:** The reaction product should be colored or electron scattering, so that it can be visualized easily with a light or electron microscope.
- **Insolubility of the Reaction Product:** The reaction product should be insoluble, so that it remains in close proximity to the substance it marks.

3. Gingiva

Gingiva compromises of epithelium, basement membrane and lamina propria.

Gingival epithelium consists of keratinocytes, non-keratinocytes and enzymes.

3.1 Immunohistochemistry of gingival epithelium

Proteins

Proteins recognized in epithelium are cytokeratins, keratolinin, in volucrin and flaggrin.

Keratins

- Keratins consists of different polypeptide subunits, High sulfhdyrl and disulphide content
- With performic acid- Alcian blue technique- the disulphide stains BLUE.
- Stains Red with H&E
- Keratin retains the red phloxine stain avidly, so Lendrums Phloxine Tartrazine Technique is suitable method for its demonstration.
- Keratohyalin granules stain deep blue with H & E stain.

Cytokeratins

Gingival epithelium is characterized by intermediate filament called cytokeratins, responsible for maintenance of homeostasis of keratinocytes.

Five classes of intermediate filaments have been described

1. Acidic cytokeratins and
2. Basic cytokeratins are cytoplasmic intermediate filaments.
3. Vimentin, Desmin,
4. Neurofilaments and
5. Nuclear lamins are nuclear intermediate filaments.

Acidic (type I) Cytokeratins include CK10, CK12, CK13, CK14, CK16, CK17, CK18, CK19 and CK20.

Basic (type II) Cytokeratins include CK1, CK2, CK3, CK4, CK5, CK6, CK7, CK8 and CK9.

Cytokeratins are numbered in sequence contrary to their molecular weight eg lower molecular weight keratins K19.

They occur in pairs of combination of type I and type II. Absence of pairs is susceptible to degeneration by proteases.

Cytokeratins on one hand interact with nuclear membrane and on other hand connect to plasma membrane via desmosomes and hemi desmosomes.

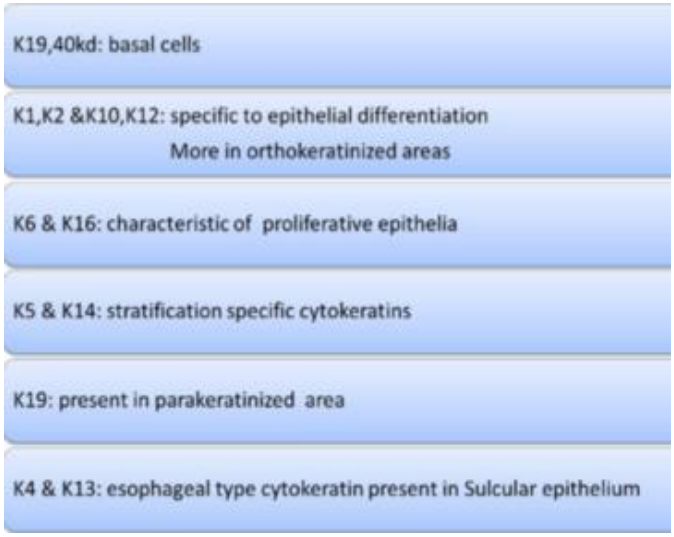
Interaction with membrane proteins are mediated by members of plakin family like desmoplakin, plectin and some integrins.

Major function of CK is to impart structural integrity to the cell.

Cytokeratins show layer and tissue specificity.

Lower molecular weight keratins are synthesized by basal cells and higher mol wt keratins by cells as they migrate to surface.

K1 keratin polypeptide 68 kd is main component of stratum corneum (Clausen H 1986). [1]



Filaggrin

Filaggrin is a keratin binding protein present in lower layers of stratum corneum, which plays important role in barrier function [2].

It is synthesized as a large precursor, profilaggrin that undergoes specific processing during keratinocyte differentiation to produce filaggrin and is initially stored in keratohylin granules.

It forms the matrix of most differentiated epithelial cell, the corneocyte.

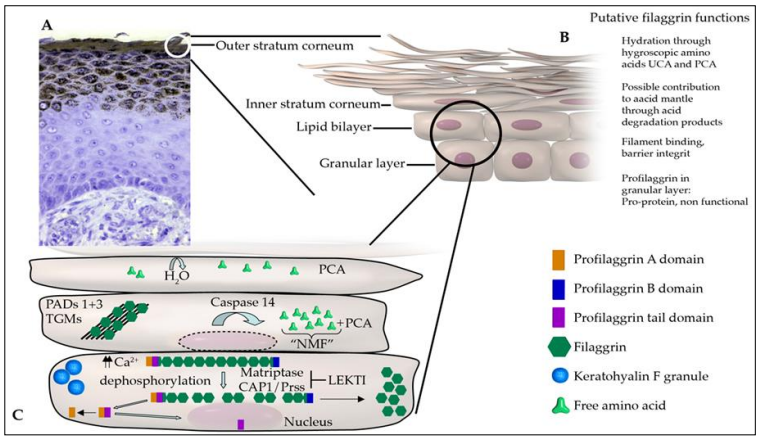


Fig 1: In middle and upper layers of stratum corneum, filaggrin is degraded into free aminoacids which have several functions including water adsorption.

Keratolinin and Involucrin

These are precursors of chemically resistant structure, envelope located below cell membrane and are produced during maturation process. Involucrin is synthesized in stratum spinosum and cross linked in stratum granulosum by transglutaminase enzyme that makes it highly stable thus providing structural support to the cell, thereby allowing the cell to resist invasion by microorganisms. [3]

Cytoplasmic Organelles and Enzymes

Cytoplasmic organelle concentration varies among different epithelial strata.

- **Deep strata**

Mitochondria are more numerous in deeper strata. ALTMANS ACID FUCHSIN- PICRIC ACID technique is used for mitochondria in which mitochondria stains RED and back ground tissue yellow.

Histochemical demonstration of succinic dehydrogenase, nicotinamide-adenine dinucleotide, cytochrome oxidase, and other mitochondrial enzymes revealed a more active tricarboxylic cycle in basal and parabasal cells, where the proximity of the blood supply facilitates energy production through aerobic glycolysis.

- **Superficial layers**

The uppermost cells of the stratum spinosum contain numerous dense granules, *keratinosomes* or *Odland bodies*, which are modified lysosomes. They contain a large amount of acid phosphatase, an enzyme involved in the destruction of organelle membranes, which occurs suddenly between the granulosum and corneum strata and during the intercellular cementation of cornified cells.

Thus acid phosphatase is another enzyme closely related to the degree of keratinization [4, 5, 6].

Acid phosphatase can be detected by The Gomori lead method: black color of acid phosphatase activity. By -Azo dye method –red color

Enzymes of the pentose shunt (an alternative pathway of glycolysis), such as glucose-6-phosphatase, increase their activity towards the surface. This pathway produces a larger amount of intermediate products for the production of ribonucleic acid (RNA), which in turn can be used for the synthesis of keratinisation proteins. This histochemical pattern is in accordance with the increased volume and amount of tonofilaments observed in cells reaching the surface, and the intensity of activity is proportional to the degree of differentiation [7, 8, 9, 10].

SD content is greater in attached gingiva than in all other zones. Its concentration decreases from basal to superficial layer. This indicates a reduction in oxidative activity in superficial layers. It may mean that these epithelial areas require less energy release due to lowered metabolic requirements.

G6PD has its maximum content in epithelium of marginal gingiva and lower content in oral mucosal epithelium, crevicular epithelium and JE. This indicates need for higher energy releases in areas of heavier keratinization & greater mechanical requirements.

- **Glycogen**

Glycogen can accumulate intra cellular when it is not completely degraded by any of Glycolytic pathways. Thus its concentration in normal gingiva is inversely related to the degree of keratinization and inflammation. [11, 12, 13, 14]

Stained by MacManus PAS method.

Some researchers consider it to be normal component of epithelium; others find it only in acanthosis, usually associated with inflammation. [15]

- **Nucleic acid**

RNA is present in large quantities in basal cells and in lowest concentration in crevicular epithelium. [16]

DNA -normally present in the nucleus of all gingival cells and is increased in gingival hyperplasia.

DNA and RNA activity of the epithelium at the gingival margin and junctional epithelium is greater than the remaining oral mucosa. [17]

Feulgen's reaction is used for DNA -hydrolysis of DNA by hydrochloric acid this process leads to formation of aldehyde groups. Free aldehyde groups react with Schiff's reagent resulting in insoluble red substance.

RNA rich organelles are stained with basic dyes ie; toluidine blue, methylene blue.

Sulfhydryl and disulfides

Sulfhydryls and disulfides are normal components of gingival epithelium [18]. In keratinization process sulfhydryls are oxidized to disulfides and both are significant in a wide range of biological activities such as enzymatic and antibody reaction, cell growth and division and cell permeability and detoxification. sulfhydrls and disulfides are present throughtout the gingival epithelium, the former is increased in keratinized and parakeratinized layers[18] and later in surface keratinized cells [19].

- **Non keratinocytes**

No keratinocyte cells are present in gingival epithelium as in other malpighian epithelia and include Melanocytes. Langerhans cells, Inflammatory cells and Merkel cells.

Melanocytes

Melanocytes aredendritic cells located in the basal and spinous layers of the gingival epithelium (Fig 2). They synthesize melanin in organelles called *premelanosomes* or *melanosomes* [20, 21, 22]. These contain tyrosinase, which hydroxylates tyrosine to dihydroxy phenylalanine (dopa), which in turn is progressively converted to melanin. Melanin granules are phagocytosed and contained within other cells of the epithelium and connective tissue, called *melanophages* or *melanophores.* Stained by Masson- Fontana method- imparts it black color.

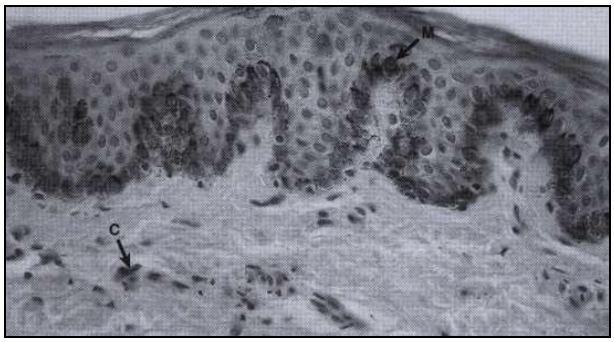


Fig 2: Melanocytes located in basal and spinous layers

- **Langerhans Cells**

Langerhans cells are dendritic cells located among keratinocytes at all suprabasal levels (Fig. 3). They belong to the mononuclear phagocyte system (reticuloendothelial

system) as modified monocytes derived from the bone marrow. They contain elongated granules and are considered macrophages with possible antigenic properties [23]. Langerhans cells have an important role in the immune reaction as antigen-presenting cells for lymphocytes. They contain g-specific granules (Birbecks' granules) and have marked adenosine triphosphatase activity. They are found in oral epithelium of normal gingival and in smaller amounts in the sulcular epithelium; they are probably absent from the junctional epithelium of normal gingiva. It stains with: Gold chloride, ATPase, Immuno florescent markers.

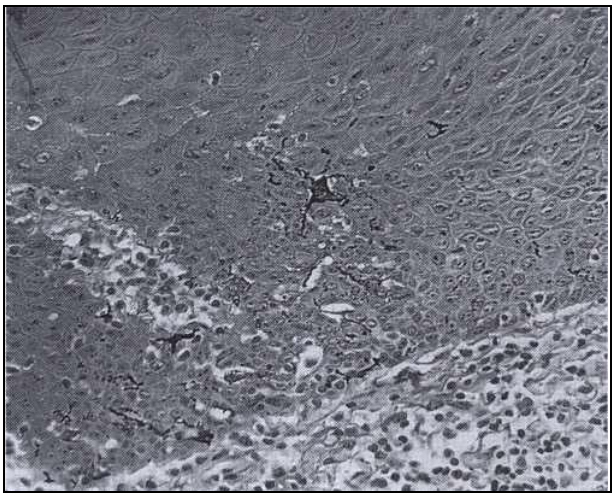


Fig 3: *Langerhans cells* are dendritic cells located among keratinocytes at all suprabasal levels

Merkel Cell *Merkel cells* are located in the deeper layers of the epithelium, harbor nerve endings, and are connected to adjacent cells by desmosomes (fig 4). They have been identified as tactile preceptors. Stained by PAS stain.

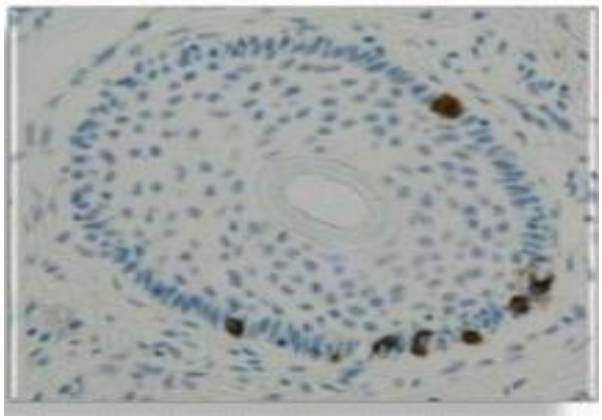


Fig 4: *Merkel cells* are located in the deeper layers of the epithelium

Basement Membrane

- The epithelium is joined to the underlying connective tissue by a *basal lamina 300* to 400 A thick, lying approximately 400 A beneath the epithelial basal layer [24]. The basal lamina consists of lamina lucida and lamina densa. Hemidesmosomes of the basal epithelial cells about the lamina lucida, which is mainly composed of the glycoprotein laminin. The lamina densa is composed of type IV collagen. The basal lamina, clearly distinguishable at the ultrastructural level, is connected to a reticular condensation of the underlying connective tissue fibrils (mainly collagen type IV) by the anchoring fibrils. The complex of basal lamina and fibrils is the periodic acid shiff [PAS] positive and agyrophillic line observed at the optical level (fig 5).

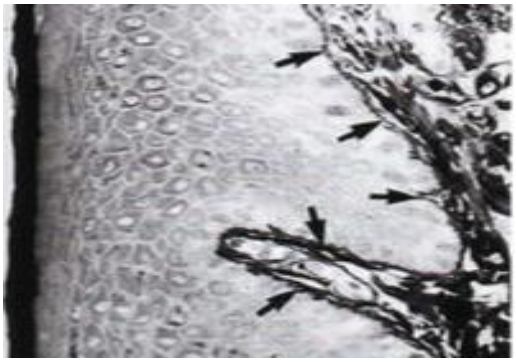


Fig 5: The complex of basal lamina and fibrils is the periodic acid shiff [PAS] positive and agyrophillic line observed at the optical level.

- **Periodic-acid methenamine silver microwave method**: stains BM-black and background- green.

Lamina Propria

Lamina propria consists of Fibers and Ground substance.

Fibers

The three types of connective tissue fibers are collagen, reticular, and elastic.

Collagen

- Collagens are the most abundant biochemical components in gingival connective tissue, provide >60% of total tissue protein.
- These fibers are organized into several characteristic and architecturally distinct units that are classified into various groups based on their location, origin, insertion. [schluger *et al* 1990]
- Dentogingival
- Dentoperiosteal
- Alveologingival Circular
- Other fibers include
- Semicircular
- Transgingival
- Intergingival
- Transseptal

Gingival collagen fibers like those fibers of other connective tissue are made up of heterotypic mixtures of collagen types, of which type 1 is major species [Bartold and Narayanan 1996; Narayanan and Page 1983].

These fibers are arranged in two patterns of organization, either as Large, dense bundles of thick fibers, or in loose pattern of short thin fibers mixed with fine reticular network. [Narayanan *et al* 1985, Rao *et al* 1979]

They principally contain type1 and type III collagen, type1 is preferentially organized into denser fibrils within the lamina propria.

Type III appears to be localized mostly as thinner fibers and distributed in a reticular pattern near the basement membrane adjacent to epithelial junction [Narayanan *et al* 1985; Romanos *et al* 1992; wang *et al* 1980]

Table 1: Gingival fiber arrangement

Name	Distribution
Dentogingival fibers	Inserted into the root surface as Sharpey's fibers and fan out from the root surface subjacent to the junctional epithelium and coronal to the alveolar crest into the gingival tissues
Dentoperiosteal fibers	Inserted into the root surface and run over the alveolar crest and insert into the buccal lingual periosteum
Alveolgingival fibers	Arise at the alveolar crest and fan out into the free and attached gingivae
Circular and semicircular fibers	Are located coronal to the transseptal fibers and run in a circumferential or semicircular manner around the teeth
Transgingival and intergingival fibers	Closely related to the semicircular fibers. Arise in the cementum and splay through the interdental septum and eventually coalesce with the semicircular fibers of the adjacent tooth
Interpapillary fibers	Run in a buccolingual direction through the interdental tissue
Periosteogingival fibers	Inserted into the alveolar periosteum and splay out into the attached gingiva
Intercircular fibers	Located both bucally and lingualy and run in a mesiodistal manner to join circular fibers of adjacent teeth
Transseptal fibers	Arise in the cementum and traverse the interdental alveolar crest and reinsert in the cementum of the adjacent tooth

Type 5 collagen in healthy gingiva accounts for less than 1% of total collagen. Immunostaining has revealed that this collagen distributes throughout the tissues in parallel filamentous pattern and appears to coat denser fibers composed of type1 and type III collagens [Narayanan *et al* 1985; Romanos *et al* 1991a, 1991b]

Staining

- Type 1 collagen (fig 6) stains strongly with acid dyes, due to the affinity of cationic groups of proteins for anionic reactive groups of acid dyes.
- They may be demonstrated more selectively by compound solutions of acid dyes eg: van Gieson or by sequential combinations of acid dyes eg: Massons trichrome, Lendrum's MSB etc.

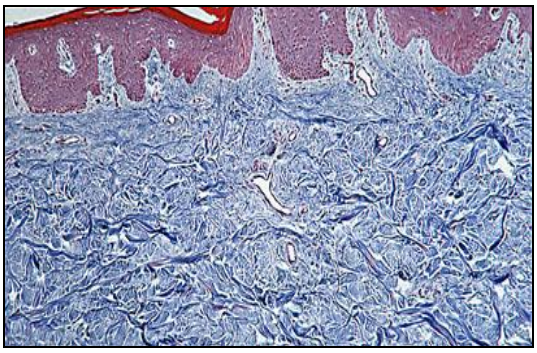


Fig 6: Type 1 collagen stains strongly with acid dyes

Reticular fibers

- Reticular fibers (fig 7) may be demonstrated distinctly in praffin sections, using one of many argyrophil –type silver impregnation techniques available or in frozen section, by the periodic acid schiff technique.
- Both methods of demonstration are dependent upon the reactive groups present in the carbohydrate matrix not upon the fibrillar elements of the fiber.
- Reticulin fibers are present at epithelial connective tissue and endothelial connective tissue interface.

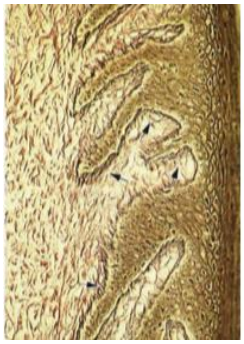


Fig 7: Reticular fibers may be demonstrated distinctly in praffin sections

Elastic fiber system

- oxytalan
- Elastin
- Elaunin

Staining for elastic fibers: _Verhoff's method-Black color _Resorcin -fuchsin method-Brown to purple _Methyl violet/ethyl violet-resorcin method for elastic fibers-Blue-Black.

Oxytalan fibers

Oxytalan fibers (fig 8) are scarce in gingiva. Long thin fibrils with diameter of approximately 150A. These connective tissue fibers can be demonstrated light microscopically only after previous oxidation with peracetic acid, potassium permagnate and performic acid.

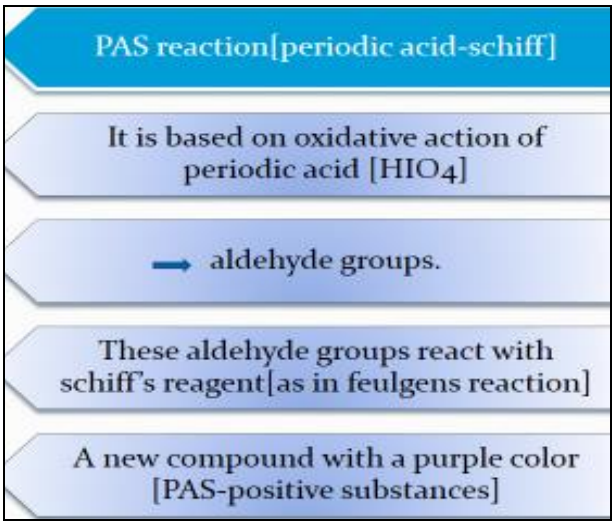
Fig 8: Oxytalan fibers are scarce in gingiva

- **Elaunin**

Stains with orcein, aldehyde fuchsin and resorcin-fuchsin without prior oxidation.

Ground substance

The ground substance fills the space between fibers and cells, is amorphous, and has a high content of water. It is composed of proteoglycans, mainly hyaluronic acid and chondroitin sulfate, and glycoproteins, mainly fibro nectin. Glycoproteins account for the faint pas positive reaction of the ground substance



Proteoglycans

The major glycosaminoglycans of gingival connective tissue is dermatan sulfate which accounts for 60% of total gingival glycosaminoglycans whereas heparin sulfate accounts for 5% approximately.

Dermatan sulfate is widespread in gingival connective tissues; however it shows a predilection to stain intensely in the tissues immediately subjacent to epithelium, there it is associated with the d-band of collagen fibers,and it is believed to provide stability to collagen fibers. [Erlinger *et al* 1995]

In deeper connective tissues of gingiva dermatan sulfate is present on collagen fibers along with chondroitin sulfate [Bartold 1992] and it is also located in the perivascular areas associated with most blood vessels. [Shibutani *et al*]

Heparan sulfate is largely found within the basement membranes, on cell surfaces and in capillary endothelium, where it forms a network {Shibutani *et al* 1989}

Proteoglycans species identified in gingiva include decorin, biglycan, versican and syndecan [Bratt *et al* 1992; larjava *et al* 1992a].

Decorin: major proteoglycan localized on collagen fiber bundles in subepithelial region [Hakkinen *et al* 1993].

Biglycans: forms fine filamentlike structures in epithelium and connective tissue.

CD44: cell surface proteoglycan located on fibroblasts and at cell to cell contact areas at basal and spinous areas of oral epithelium. [Hakkinen *et al* 1993].

Blood vessels

Blood vessels (fig 9) are easily evidenced in tissue sections by means of immunohistochemical reactions against proteins of endothelial cells [factor VIII and adhesion molecules]

Before these techniques were developed, vascularization patterns of periodontal tissues had been described using histoenzymatic reactions for alkaline phosphatase and adenosine triphosphatase because of the great activity of these enzymes in endothelial cells [25].

In exprimental animals the perfusion with Indian ink also was used to study vascular distribution.

The injection and subsequent demonstration of peroxidase allow blood vessel identification and permeability studies. [26]

The PAS reaction also outlines vascular walls by a positive line in their basal membrane [27].

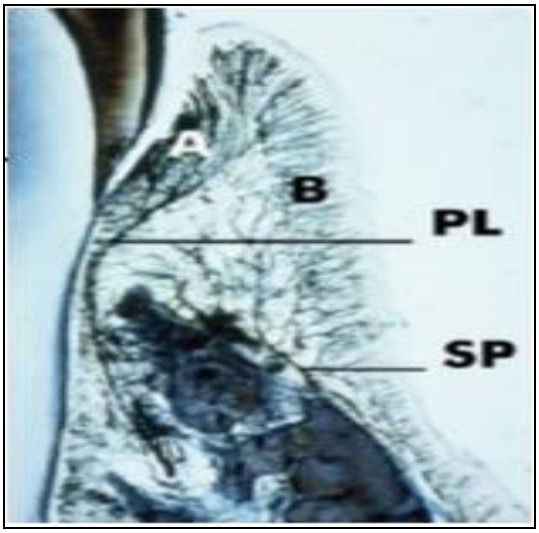


Fig 9: Blood vessels are easily evidenced in tissue sections by means of immunohistochemical reactions

Enzymes

Alkaline phosphatase is present in endothelial cells, in capillary walls and possibly in fibers of connective tissue. It has been described in keratinized and parakeratinized surface layers but some doubt that it occurs in epithelium.

Acid phosphatase found in epithelium in greatest concentration in surface and prickle cell layers is related to keratinisation.

Diphospho and triphospho pyridine nucleotide reductase present in all epithelial cells except keratin and parakeratin, in desmosomes, tonofibrils and nucleoli, suggest oxidative metabolic pathway for the formation of the keratin precursor substance and keratin.

Acetyl cholinesterase and non-specific cholinesterase are present in gingival connective tissue.

Endogenous reducing enzymes succinic dehydrogenase, glucose-6-phosphate dehydrogenase, lactic dehydrogenase, beta D glucuronidase, beta glucosidase, beta galactosidase and amino peptidase have been observed in gingival.

Esterase occurs in basal and granular layers of epithelium and in connective tissue near periodontal pockets.

Collagenase is produced in epithelium and connective tissue of normal gingival as well as in periodontal ligament and alveolar bone.

Cytochrome oxidase activity occurs in sulcal and attachment epithelium in basal layers of marginal and attached gingiva and connective tissue.

5 nucleotidase occurs in blood vessels and surface epithelial cells of keratinized gingival and only in blood vessels of non-keratinized and parakeratinized gingival [28].

The oxygen consumption of normal gingival (QO_2 1.6 ± 0.37) is comparable to that of skin (QO_2 1.48 ± 0.48) [29]. The respiratory activity of epithelium is approximately three times greater than that of the connective tissue [30] and sulcal

epithelium is approximately twice that of whole gingival [31].

Conclusion

Histochemical techniques provide useful information regarding the chemical and enzyme systems of normal gingiva. In addition to adding to our understanding of physiologic processes in the gingiva this information provides the guidelines for interpreting the changes which occur in gingival diseases.

Footnotes

Source of Support: Nil
Conflict of Interest: None declared.

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